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Cell-permeable, Mitochondrial-targeted, Peptide Antioxidants

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ABSTRACT

Cellular oxidative injury has been implicated in aging and a wide array of clinical disorders including ischemia-reperfusion injury; neurodegenerative diseases; diabetes; inflammatory diseases such as atherosclerosis, arthritis, and hepatitis; and drug-induced toxicity. However, available antioxidants have not proven to be particularly effective against many of these disorders. A possibility is that some of the antioxidants do not reach the relevant sites of free radical generation, especially if mitochondria are the primary source of reactive oxygen species (ROS). The SS (Szeto-Schiller) peptide antioxidants represent a novel approach with targeted delivery of antioxidants to the inner mitochondrial membrane. The structural motif of these SS peptides centers on alternating aromatic residues and basic amino acids (aromatic-cationic peptides). These SS peptides can scavenge hydrogen peroxide and peroxynitrite and inhibit lipid peroxidation. Their antioxidant action can be attributed to the tyrosine or dimethyltyrosine residue. By reducing mitochondrial ROS, these peptides inhibit mitochondrial permeability transition and cytochrome *c* release, thus preventing oxidant-induced cell death. Because these peptides concentrate >1000-fold in the inner mitochondrial membrane, they prevent oxidative cell death with EC_{50} in the nM range. Preclinical studies support their potential use for ischemia-reperfusion injury and neurodegenerative disorders. Although peptides have often been considered to be poor drug candidates, these small peptides have excellent "druggable" properties, making them promising agents for many diseases with unmet needs.

KEYWORDS: reactive oxygen species, free radicals, mitochondrial permeability transition, apoptosis, ischemia-reperfusion injury

INTRODUCTION

Reactive oxygen species (ROS) can damage cells by oxidizing membrane phospholipids, proteins, and nucleic acids. These damaging effects of ROS are normally kept under control by endogenous antioxidant systems including glutathione, ascorbic acid, and enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase. Oxidative stress occurs when antioxidant systems are overwhelmed by ROS, and the resulting oxidative damage can lead to cell death.

SOURCES OF ROS

NADPH oxidase on the plasma membrane, and cytoplasmic enzymes such as xanthine oxidase and nitric oxide synthase, can all generate superoxide anion ($O_2^{\bullet -}$). In addition, mitochondria are a major source of ROS[1] (see Figure 1). Superoxide anion is produced by the electron transport chain on the inner mitochondrial membrane, and the rate of production is dependent on mitochondrial potential. In the presence of mitochondrial SOD, $O_2^{\bullet -}$ can be converted to hydrogen peroxide (H_2O_2), which can then diffuse out of mitochondria into the cytoplasm. In the presence of high iron concentrations, H_2O_2 can form the highly reactive hydroxyl radical ($OH^\bullet$) via the Fenton reaction. $O_2^{\bullet -}$ can also react with nitric oxide to form the highly reactive peroxynitrite ($ONOO^-$).

CONSEQUENCES OF REACTIVE OXYGEN AND REACTIVE NITROGEN SPECIES

Reactive oxygen and reactive nitrogen species can cause damage to all cellular macromolecules, including nucleic acids, proteins, carbohydrates, and lipids. Membrane lipids are major targets of ROS, and lipid peroxidation may lead to membrane dysfunction and alterations in cell permeability.

Mitochondria are particularly vulnerable to oxidative damage because they are constantly exposed to high levels of ROS (Figure 2). Mitochondrial DNA has been shown to undergo oxidative damage. In addition to lipid peroxidation, protein oxidation and nitration results in altered function of many metabolic enzymes in the mitochondrial matrix as well as those comprising the electron transport chain. A particularly relevant protein that loses function upon oxidation is SOD, which would further compromise antioxidant capacity and lead to further oxidative stress.

Increasing evidence suggests that ROS play a key role in promoting cytochrome *c* release from mitochondria.[2]

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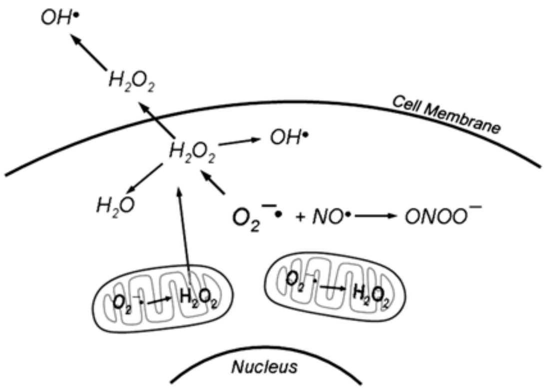

Figure 1. Formation of intracellular reactive oxygen and nitrogen species.

Cytochrome *c* is normally bound to the inner mitochondrial membrane by association with cardiolipin.[3] Peroxidation of cardiolipin leads to dissociation of cytochrome *c* and its release through the outer mitochondrial membrane into the cytosol.[4] The mechanism by which cytochrome *c* is released through the outer membrane is not clear. One mechanism may involve mitochondrial permeability transition (MPT), with swelling of the mitochondrial matrix and rupture of the outer membrane (Figure 3). ROS may promote MPT by causing oxidation of thiol groups on the adenine nucleotide translocator, which is believed to form part of the MPT pore.[5] Cytochrome *c* release may also occur via MPT-independent mechanisms and may involve an oligomeric form of Bax[6] (Figure 3). Cytochrome *c* in the cytoplasm triggers the activation of caspase-9, which triggers the caspase cascade and ultimately leads to apoptosis.[7,8]

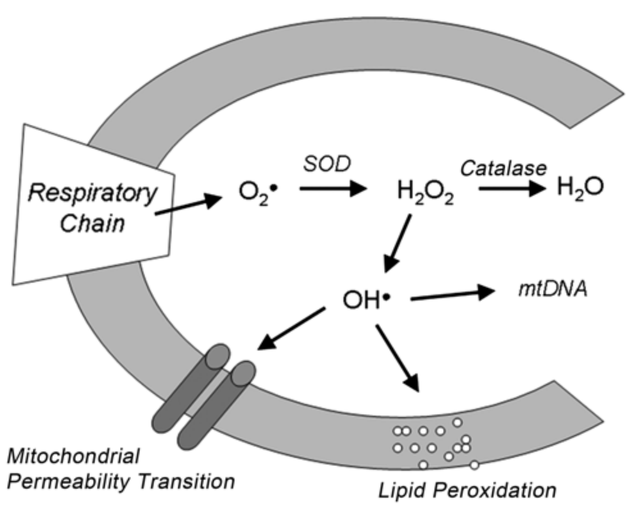

Figure 2. Mitochondrial damage caused by reactive oxygen and nitrogen species. Free radicals generated by the electron transport chain can result in oxidative damage to mitochondrial DNA and proteins, lipid peroxidation, and opening of the mitochondrial permeability transition pore.

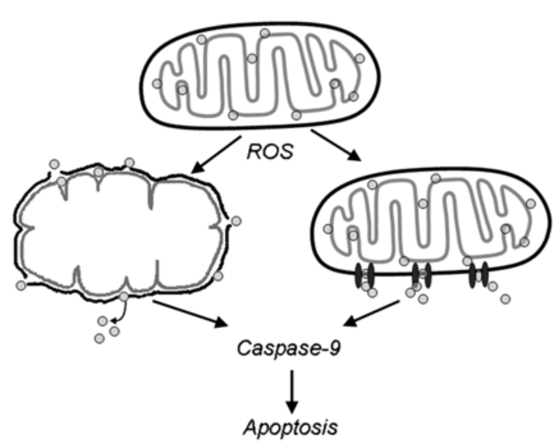

Figure 3. Cytochrome *c* release from mitochondria. Cytochrome *c* (○) is normally associated with cardiolipin on the inner mitochondrial membrane. Cytochrome *c* is dissociated upon oxidation of cardiolipin and is believed to be released out of mitochondria either by mitochondrial permeability transition resulting in mitochondrial swelling and rupture of the outer membrane, or by channels formed by oligomerization of Bax. In the cytoplasm, cytochrome *c* activates caspase-9 and promotes apoptosis.

Diseases Associated With Oxidative Stress

Cellular oxidative injury is implicated in aging and a wide array of clinical disorders including ischemia-reperfusion injury, neurodegenerative diseases, diabetes, and inflammatory diseases such as atherosclerosis, arthritis, and hepatitis. Oxidative damage is also believed to play a role in drug-induced toxicity, including acetaminophen hepatotoxicity, methamphetamine neurotoxicity, cocaine hepatotoxicity, alcoholic fatty liver, and anthracycline toxicity.

Search for Effective Antioxidants

Large doses of antioxidants have been found to be effective in many animal models of diseases associated with oxidative damage. However, clinical trials with antioxidants such as vitamin E or recombinant human SOD have generally failed to demonstrate significant benefits, leading to the proposed antioxidant paradox.[9-11] One possible reason is that many of the antioxidants can also have prooxidant activity besides antioxidant activity, especially in the presence of transitional metals.[12] Another possibility is that some of the antioxidants do not reach the relevant sites of free radical generation. Large proteins such as SOD do not penetrate cell membranes and are therefore ineffective against intracellular ROS. Antioxidants such as vitamin E and coenzyme Q are very lipophilic and tend to be retained in cell membranes. The ideal antioxidant should be cell-permeable and be able to target mitochondria where they can protect mitochondria against oxidative damage. The conjugation of a triphenylalkylphosphonium cation to lipophilic antioxidants such as coenzyme Q (MitoQ) and vitamin E has been used to promote their delivery into mitochondria by taking advantage of the potential gradient across the inner mitochondrial membrane.[13] However, high concentrations of MitoQ have been shown to cause mitochondrial depolarization.[14,15]

Discovery of Cell-permeable, Mitochondria-targeted Peptide Antioxidants

A series of small, cell-permeable, mitochondria-targeted, antioxidant peptides that can protect mitochondria from oxidative damage was recently reported[16] (see Table 1).

Design for Free Radical Scavenging Properties

The structural motif of these SS (Szeto-Schiller) peptides centers on alternating aromatic residues and basic amino acids (aromatic-cationic peptides).[16] These SS peptides can scavenge H_2O_2 and $ONOO^-$ and inhibit lipid peroxidation in vitro as demonstrated by the inhibition of linoleic acid oxidation and low-density lipoprotein (LDL) oxidation (Figure 4). Their antioxidant action can be attributed to the tyrosine, or dimethyltyrosine (Dmt), residue. Tyrosine can scavenge oxyradicals forming relatively unreactive tyrosyl radicals, which can be followed by radical-radical coupling to give dityrosine, or react with superoxide to form tyrosine hydroperoxide.[17] Dimethyltyrosine is more effective than tyrosine in scavenging of ROS. The specific location of the tyrosine or dimethyltyrosine residue is not important as SS-31 was found to be as effective as SS-02 in scavenging H_2O_2 and inhibiting LDL oxidation. However, replacement of Dmt with phenylalanine in SS-02 (SS-20) eliminated the scavenging ability (Figure 4).

Method of Cell Uptake

These small peptides contain an amino acid sequence that allows them to freely penetrate cells despite carrying a 3+ net charge at physiologic pH.[18] These aromatic-cationic peptides are taken up into cells in an energy-independent nonsaturable manner. Uptake studies with [^{3}H]SS-02 showed rapid uptake with steady-state achieved in less than 30 minutes.[16] This finding suggests that these peptides can freely pass through the plasma membrane in both directions. Unlike the larger cationic peptides such as *Tat* peptide,[19,20] there is no evidence of vesicular localization that would result from endocytosis.

Table 1. Cell-permeable, Mitochondria-targeted Peptides

SS-01	H-Tyr-D-Arg-Phe-Lys-NH_2
SS-02	H-Dmt-D-Arg-Phe-Lys-NH_2
SS-31	H-D-Arg-Dmt-Lys-Phe-NH_2
SS-20	H-Phe-D-Arg-Phe-Lys-NH_2

Method of Mitochondria Targeting

These SS peptides have a sequence motif that targets them to mitochondria. Figure 5 shows the internalization and targeting of a fluorescent peptide analog (Dmt-D-Arg-Phe-atnDap-NH_2; atn = β-anthraniloyl-L-α,β-diaminopropionic acid) to mitochondria in living cells. The confocal images show that the pattern of localization of the fluorescent peptide analog is identical to that of Mitotracker TMRM, a fluorescent dye that is taken up into mitochondria in a potential-driven manner. Incubation of isolated mitochondria with [^{3}H]SS-02 confirmed that it is taken up and concentrated >1000-fold in mitochondria.[16] Contrary to MitoQ, the uptake of these aromatic-cationic peptides into mitochondria is not dependent on mitochondrial potential, and they are localized to the inner mitochondrial membrane rather than in the matrix (see schematic in Figure 5).

Inhibition of Oxidative Cell Death

By targeting and partitioning in the inner mitochondrial membrane, these SS peptides are extremely potent in preventing oxidative cell death. *Tert*-butylhydroperoxide (*t*BHP) is a membrane-permeant oxidant compound that can induce cell death via apoptosis or necrosis.[21,22] *t*BHP is cell permeable and can generate *t*-butoxyl radicals via the Fenton reaction, resulting in lipid peroxidation and depletion of intracellular glutathione, followed by modification of protein thiols and loss of cell viability. Treatment of cells with *t*BHP causes rapid oxidation of pyridine nucleotides and increased ROS production in mitochondria.[23,24] The SS peptides are very potent in reducing intracellular ROS and preventing cell death after *t*BHP treatment, with EC_{50} in the nM range (Figure 6).[16] In contrast, most antioxidants require at least 100 μM to reduce oxidative cell death.[24-26] MitoQ was able to block H_2O_2-induced apoptosis at 1 μM, but >10 μM caused cytotoxicity.[15]

Protection of Mitochondria Against Oxidative Damage

Recent evidence suggest that *t*BHP-induced apoptosis is triggered by MPT.[22] Peroxidation of cardiolipin induces the dissociation of cytochrome *c* from the inner mitochondrial membrane and subsequent release into the cytoplasm as a result of the opening of the MPT pore. Calcium overload can also lead to increase in mitochondrial ROS and opening of the MPT pore. By reducing mitochondrial ROS, the scavenging SS peptides (SS-02 and SS-31) were able to inhibit MPT, prevent mitochondrial swelling, and reduce cytochrome *c* release in response to Ca^{2+} overload (Figure 7).

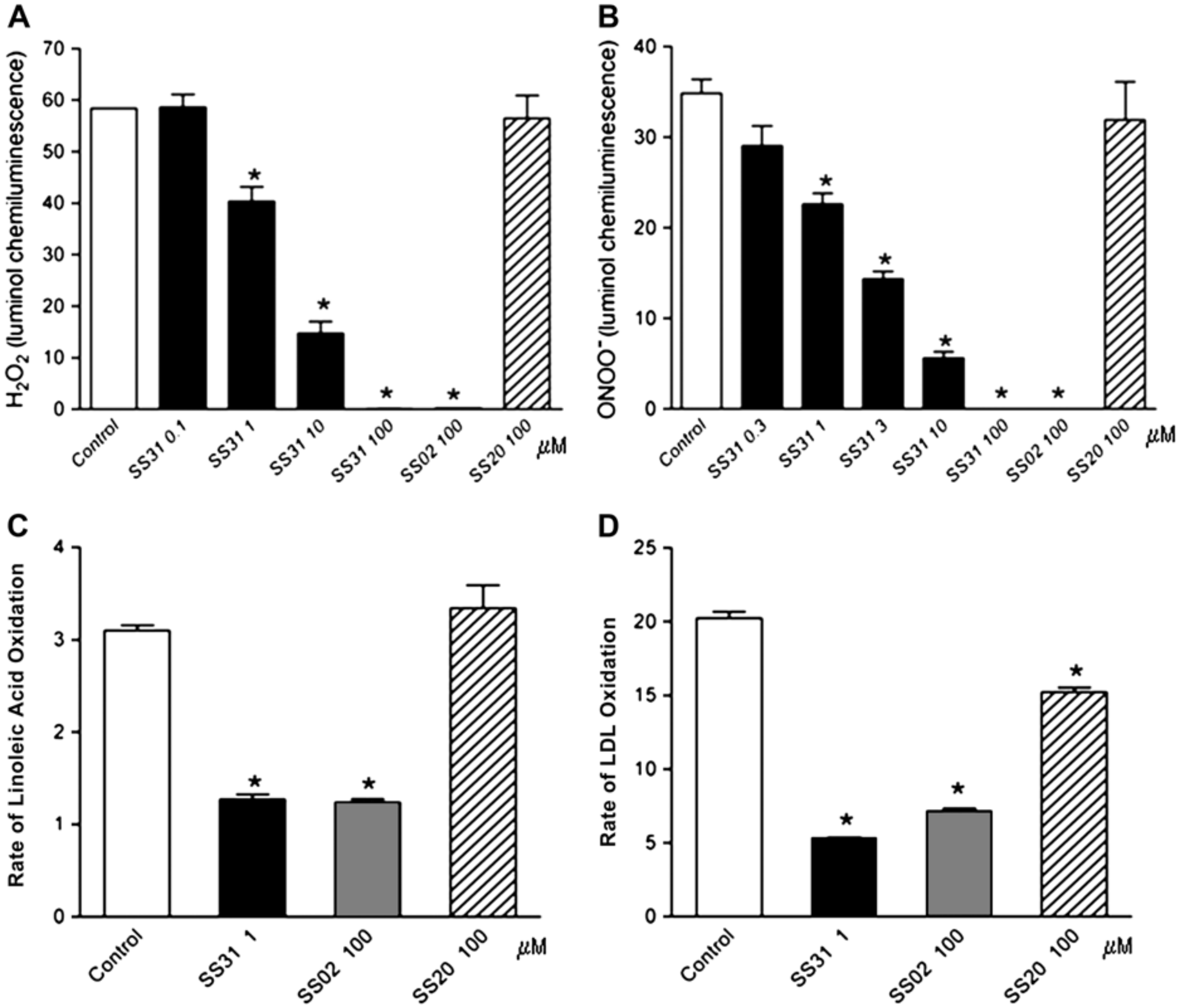

Figure 4. Antioxidant properties of SS peptides. (A) Scavenging of H_2O_2 by SS-31 and SS-02 but not SS-20. H_2O_2 was measured by luminol chemiluminescence; (B) Scavenging of $ONOO^-$ by SS-31 and SS-02 but not SS-20. $ONOO^-$ was measured by luminol chemiluminescence; (C) Linoleic acid peroxidation was inhibited by SS-31 and SS-02 but not SS-20. Linoleic acid peroxidation was initiated by 2,2'-azobis(2-amidinopropane) and detected by formation of conjugated dienes measured by absorbance at 234 nm; and (D) LDL oxidation was inhibited by SS-31 and SS-02 but not SS-20. Human LDL was oxidized by $CuSO_4$, and the formation of conjugated dienes was measured by absorbance at 234 nm.

On the other hand, the nonscavenging peptide, SS-20, did not prevent mitochondrial swelling at the same concentrations. These results support the proposal that ROS may potentiate MPT via oxidation of the adenine nucleotide translocator. The ability of SS peptides to prevent MPT will minimize MPT-induced ROS accumulation and further reduce oxidative damage on mitochondria.[27]

Protection Against Ischemia-Reperfusion Injury

ROS and mitochondrial permeability transition are thought to play a major role in ischemia-reperfusion injury.[28] In animal models, overexpression of SOD is associated with protection against reperfusion injury, while SOD knockout animals are more susceptible to reperfusion injury. However, although oxygen radical scavengers were found to be effective in several ex-vivo heart studies or animal studies, results from clinical trials have generally been disappointing. The efficacy of scavengers in preclinical studies may be attributed to their administration prior to ischemia in most studies rather than upon reperfusion as is usually done in patients with acute myocardial infarction or stroke. In addition, recombinant SOD is a large protein molecule that is unlikely to distribute across the endothelium and penetrate cells.

Small molecule SOD mimetics were found to be effective in canine studies but were associated with acute hypotension, and clinical trials of efficacy in humans have not been reported.

Both SS-02 and SS-31 were able to prevent myocardial stunning when administered upon reperfusion in the ex-vivo

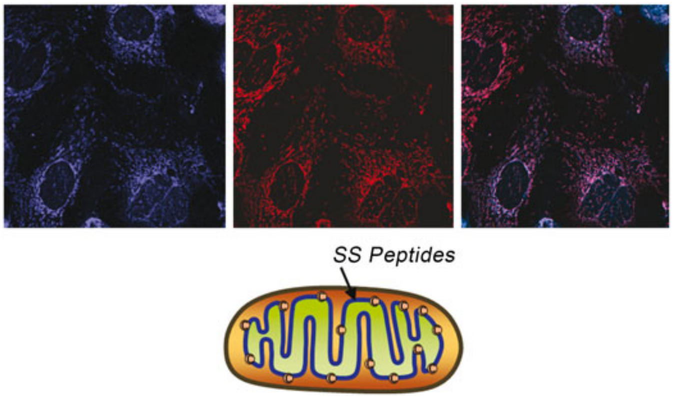

Figure 5. Internalization and targeting of fluorescent SS peptide to mitochondria in living cells. Caco-2 cells were incubated with Dmt-D-Arg-Phe-atnDap-NH_2 (blue fluorescence) and TMRM (red fluorescence) at 37°C for 30 minutes and imaged by confocal laser scanning fluorescence microscopy.[16] Overlay of the 2 images shows colocalization of the fluorescent peptide and TMRM, suggesting mitochondrial targeting.

guinea pig heart.[16,29] In contrast, SS-20, which has no scavenging ability, was unable to prevent myocardial stunning when administered upon reperfusion. The ability of SS-02 to prevent myocardial stunning has been confirmed in rats in vivo.[30] These findings with the SS peptides support the proposal that ROS play a major role in reperfusion-induced myocardial stunning.

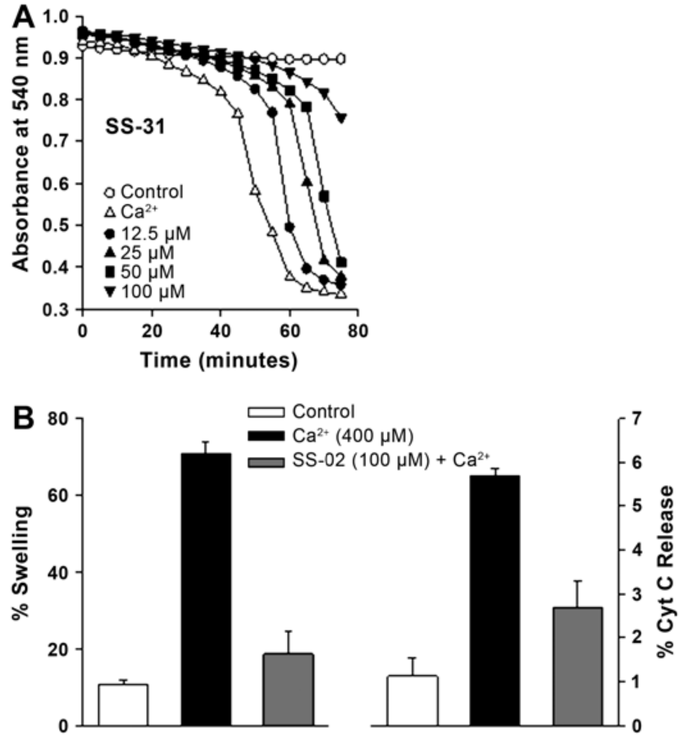

Figure 7. SS peptides inhibited mitochondrial swelling (A) and prevented cytochrome *c* release (B) from isolated mitochondria subjected to calcium overload.[16] Isolated mouse liver mitochondria were exposed to 50 μM Ca^{2+} and swelling measured by absorbance at 540 nm. The amount of cytochrome *c* released was expressed as percentage of total cytochrome *c* in mitochondria.

Pharmacokinetic Properties of SS Peptides

These antioxidant peptides are highly "druggable." They are small and easy to synthesize, readily soluble in water, and resistant to peptidase degradation. The presence of a *D*-amino acid in either the first or second position renders them resistant against aminopeptidase activity, and amidation of the C-terminus reduces hydrolysis from the C-terminus. Despite carrying 3+ net charge at physiological pH, these peptides have been shown to readily penetrate cell membranes of a variety of cell types.[18] Their cellular uptake appears to be concentration-dependent, nonsaturable, and not requiring energy.[18] In addition to cell uptake, SS-02 was shown to readily penetrate a monolayer of intestinal epithelial cells in both apical-basolateral and basolateral-apical directions. Furthermore, analgesia studies in mice suggest that SS-02 readily penetrates the blood-brain-barrier after subcutaneous administration.[31] This has also been confirmed by the detection of [^{3}H]SS-02 in mouse brain within 5 minutes after intravenous injection (Andrew Gifford, unpublished results, 2005). Finally, pharmacokinetic studies have revealed relatively long elimination half-life for SS-02 in sheep and rats.[32]

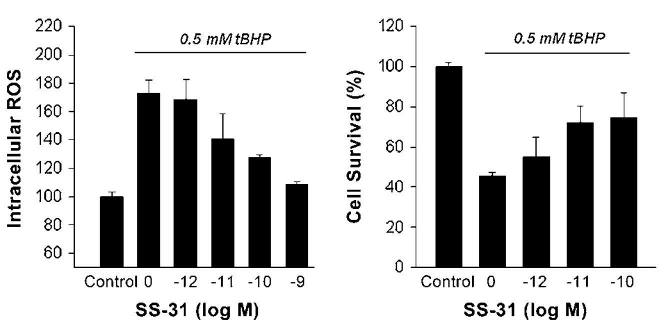

Figure 6. Dose-dependent SS-31 reduced intracellular ROS and prevented cell death caused by *t*BHP. Neuroblastoma N_2A cells were treated with 0.5 mM *t*BHP for 40 minutes. Intracellular ROS was measured by 5-(and 6)-carboxy-2'7'-dichlorohydrofluorescein diacetate, and cell death was measured by the MTT assay.

Conclusions

Oxidative damage is believed to be associated with aging and numerous degenerative diseases. However, available antioxidants have not proven to be particularly effective against many of these disorders. A possibility is that some of the antioxidants do not reach the relevant sites of free radical generation, especially if mitochondria are the primary source of ROS generation. The SS peptides represent a novel approach with targeted delivery of antioxidants to mitochondria. By protecting mitochondrial viability, these peptides can also minimize further ROS generation. By selectively concentrating in mitochondria, these peptides are extraordinarily potent in protecting against oxidative cell death. Preclinical studies support their potential use for ischemia-reperfusion injury and neurodegenerative disorders. Although peptides have often been considered to be poor drug candidates, these small peptides have excellent "druggable" properties, making them promising agents for many diseases with unmet needs.

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